AWARD NUMBER: W81XWH-05-1-0401

TITLE: Identification of Biomarkers Associated with the Healing of Chronic Wounds

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REPORT DATE: June 2009

TYPE OF REPORT: Final Addendum

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1 June 2009	Final Addendum	1 Jan 2008 – 31 May 2009

4. TITLE AND SUBTITLE

Identification of Biomarkers Associated with the Healing of Chronic Wounds

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0401

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Daemen College
Amherst, NY 1426

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
It is the objective of this study to identify the biomarkers associated with the earliest stages of healing in chronic wounds. The findings of this study are intended to facilitate the development a diagnostic tool, which could be used to evaluate the healing process. Samples have been collected from interior and peripheral wound sites over 6 weeks from 50 subjects thus far and analyzed using 2D Page, iTRAQTM, and antibody arrays. Different proteins have been identified in healed versus chronic wounds and in different locations within the wounds. The correlation of wound biochemistry, clinical appearance, and clinical outcome is critical to the understanding of pressure ulcer healing. These findings will aid in the development of criteria for evaluating the healing process and response to treatment.

15. SUBJECT TERMS
Chronic wound, healing, 2D Page, iTRAQTM, antibody arrays

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	20	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

The fact that there are differences in chronic versus normal healing wounds is well documented. What is unknown at this time are the specific biomarkers associated with healing wounds, the role each of these biomarkers play in wound healing, and the biomarkers that can serve as the earliest predictors of healing. It is our hypothesis that specific cytokines, proteases, and growth factors serve as the earliest indicators of healing in chronic wounds. It is the objective of this study to identify the biomarkers associated with the earliest stages of healing in chronic wounds. The findings of this study are intended to facilitate the development a diagnostic tool, which would assist in the evaluation of the healing process.

BODY

Statement of Work

Technical Objective 1: To identify the biochemical changes that occur as a chronic wound begins the healing process.

a. Analyze fluid samples to determine proteins present
b. Identify differences between subjects and subject time points
c. Confirm protein identities

Technical Objective 2: To assess the rate of healing of the wounds analyzed.

a. Measure wound
b. Calculate trajectories of healing for wounds over time

Technical Objective 3: To evaluate the location of the biomarkers assessed.

a. Compare proteins found in different locations using protein analysis

Technical Objective 4: To identify the earliest changing biomarkers occurring in wounds which progressed toward healing.

a. Correlate the changes in wound chemistry with the rate of healing
b. Analyze the earliest biochemical changes present

Technical Objectives 1,2,3, & 4:

As of June 14, 2008 enrollment of the study was closed. 121 subjects were screened and 50 were enrolled.

Technical Objective 1:

The analysis of wound fluid samples to determine the proteins present is nearing completion. A sample from each subject at each time point was collected and two-dimensional polyacrylamide gel electrophoresis (2D Page) was utilized to separate the proteins based on molecular weight and isolelectric point. 700 spots have been recognized. Changes in spot intensity were plotted versus time.

Spots were cut from numerous gels and sent to Midwest Bio Services, LLC for identification. Multiple proteins can be contained within a single spot. Figure 1 shows the gel and the spot sent for identification versus the spot intensity (2 gels for each sample) during the time course of the study and the wound clinical appearance. The multiple proteins contained in each spot, as well as the complex analysis of the gels and the expense of gels and mass spec identification confirms our decision, as previously reported, to shift to multiplexed isobaric tagging technology (iTRAQ™). The University of Texas Health Science Center (UTHSC) Proteomics Research Core, and specifically Dr William Dubinsky, were recommended by other researchers at the TATRC PLR in January 2007. Dr. Dubinsky has conducted all the iTRAQ analysis of our samples.

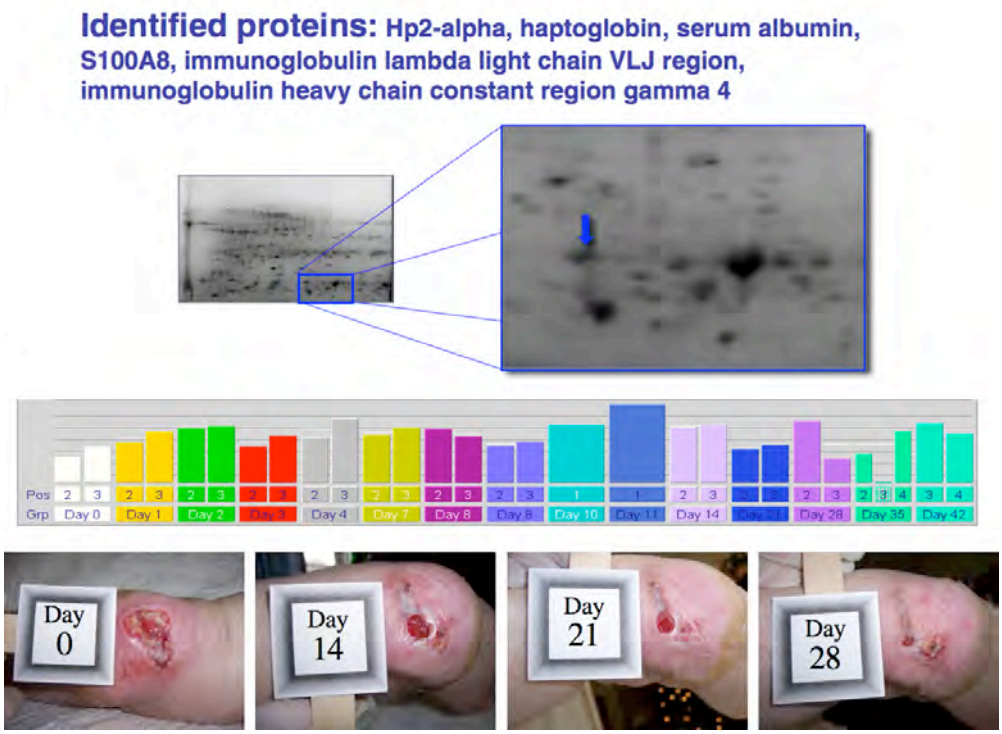


Figure 1. Spot intensity versus wound clinical appearance

iTRAQ is limited in the number of samples that can be compared in an individual analysis. Four time points are selected for analysis to determine both the proteins present and their relative changes in concentration between 4 time points. Additionally, iTRAQ identifies the most abundant proteins present and the least abundant include most cytokines, which is the majority of what has been previously reported in the literature regarding the biochemistry of pressure ulcers and other types of wounds. As a result, antibody arrays have also been run on the samples to analyze the less abundant and smaller proteins. Approximately 282 proteins have been identified in wound samples using iTRAQ. The relative change in protein amount as compared to other samples/days has been analyzed for the samples and significant differences in the proteins present have been identified. Netrophil gelatinase-associated lipocalin precursor (NGAL) was present in all wounds and Calreticulin precursor was present in chronic wounds only.

Antibody arrays have been used to identify targets for quantitative and semi-quantitative analyses. The label-based antibody array (Human L Series 507, RayBiotech, Inc.) evaluates expression levels of 507 human proteins. The greatest level of expression was seen in EN-RAGE

(S100A12), Erythropeietin, D6, EG-VEGF/PK 1, MMP-9, Progranulin, MMP-8, and TIMP-1. A Human Matrix Metalloproteinase Antibody Array (RayBiotech, Inc.) was used to examine MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-10, MMP-13, TIMP-1, TIMP-2, and TIMP-4. The Human Inflammation Antibody Array (RayBiotech, Inc.) (BLC, Eotaxin, Eotaxin-2, G-CSF, GM-CSF, I-309, ICAM-1, IFNg, IL-1a, IL-1b, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-6sR, IL-7, IL-8, IL-10, IL-11, IL-12p40, IL-12p70, IL-13, IL-15, IL-16, IL-17, MCP-1, MCSF, MIG, MIP-1a, MIP-1b, MIP-1d, PDGF-BB, RANTES, TIMP-1, TIMP-2, TNFa, TNFb, TNF RI, TNF RII) has been run on multiple samples comparing wound type and location within wounds. The MMP arrays yielded somewhat expected results. 100% of the samples had MMP-8, MMP-9, TIMP-, and TIMP-2 present and greater than 80% had MMP-1 and MMP-3. The inflammatory cytokines present in greater than 85% of the samples were TNF RI, IL-1ra, IL-1b, IL-8, IL-1a, IL-6sR, IL-5, TNF RII, IL-6. Appendix A shows the MMP/TIMP and IL-6/MMP trends over time. The overlay of this data with tissue type and wound area is very interesting. The decrease in slough and increase in granulation tissue correlates with the peak of MMP-8, MMP-9, IL-6, and IL-6 sR. These plots are being created and examined for various proteins and wound types. The analysis of these plots will continue to evaluate the trends between tissue types and wound biochemistry.

The proteins identified using iTRAQ are being analyzed using Ingenuity Pathway Analysis Software (IPA), a software package that allows analysis, modeling, and literature searches for similar proteins. The molecular and cellular function identified to have the largest number of molecules present in the wounds was cellular movement. This was the case in both healed and chronic wounds in both internal and peripheral locations. In a wound, cellular movement is necessary for healing to occur. Cell death had a large number of molecules present in chronic wounds in both the internal and peripheral locations. These molecules were not present in internal sites of healed wounds, but were present in some peripheral locations of healed wounds. This may be significant in the identification of differences in both location, as well as clinical outcome of wounds. Antigen presentation had many molecules present in IPA analysis in both the internal and peripheral locations of chronic wounds.

Analysis of regulated molecules found in the iTRAQ experiments revealed that ENO1 was down regulated in healed wounds in both internal and peripheral locations and upregulated in chronic wounds in both internal and peripheral locations. ENO1 works to create and activate collagenase and

degrades fibrin and extracellular matrix. The upregulation in chronic wounds is consistent with the role of ENO1. Also, S100A8 and S100A9 were upregulated in internal sites of chronic wounds only. These inflammatory markers and others within the S100 family are of great interest as potential markers of chronic wounds. The final set of custom antibody arrays include calreticulin precursor, several members of the S100 family, and ENO1 as targets. Final samples are being sent at this time for analysis.

Technical Objective 2:

To assess the rate of healing, the wounds were photographed and their area calculated at each time point. As previously reported, all wounds have been separated by clinical outcome into healed, healing, and chronic categories based on area measurements over the 42 days. Wounds that had a 81-100% decrease in area are categorized as healed, wounds with a 40-80% decrease in size are healing, and wounds with a less than 39% decrease in size or an increase in size were labeled chronic. Wound area versus the time point has been graphed as wound trajectories.

The amount of protein present in the wound did not correlate with wound outcome. Although the overall protein decreases as the wound heals, this is a function of the wound bed drying and sampling method versus protein present. Figure 2 shows the apparent randomness of protein present in wounds.

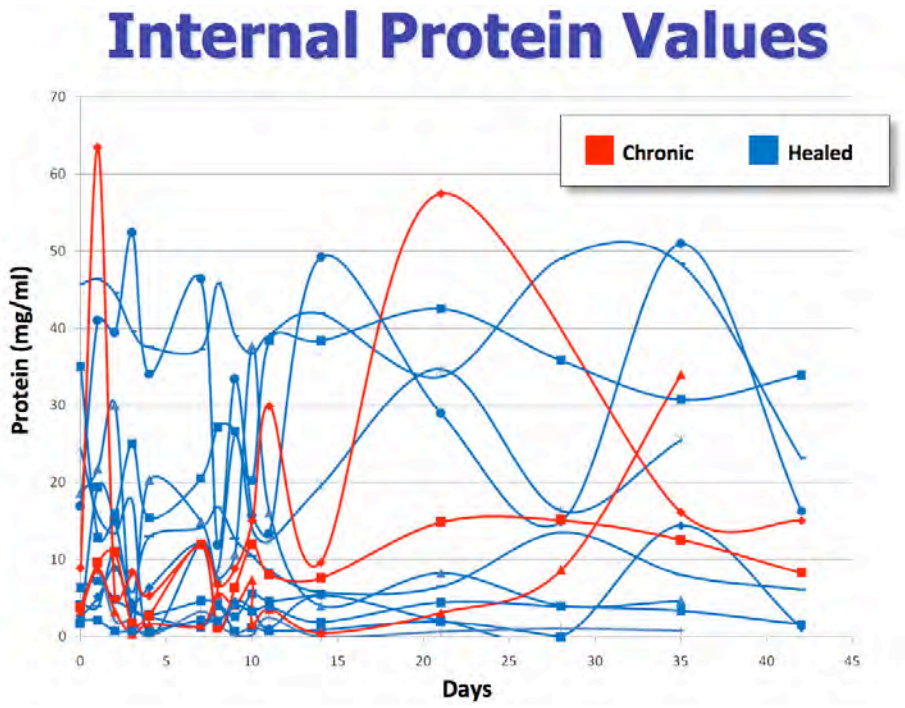


Figure 2. Protein present in chronic and healed wounds

Tissue type calculations based on the method previously reported have been completed and final correlation with clinical outcome and tissue type is being evaluated. Figure 3 demonstrates the tissue type and wound category versus protein present. Tissue type, wound area, and protein profile data are shown in Appendix A.

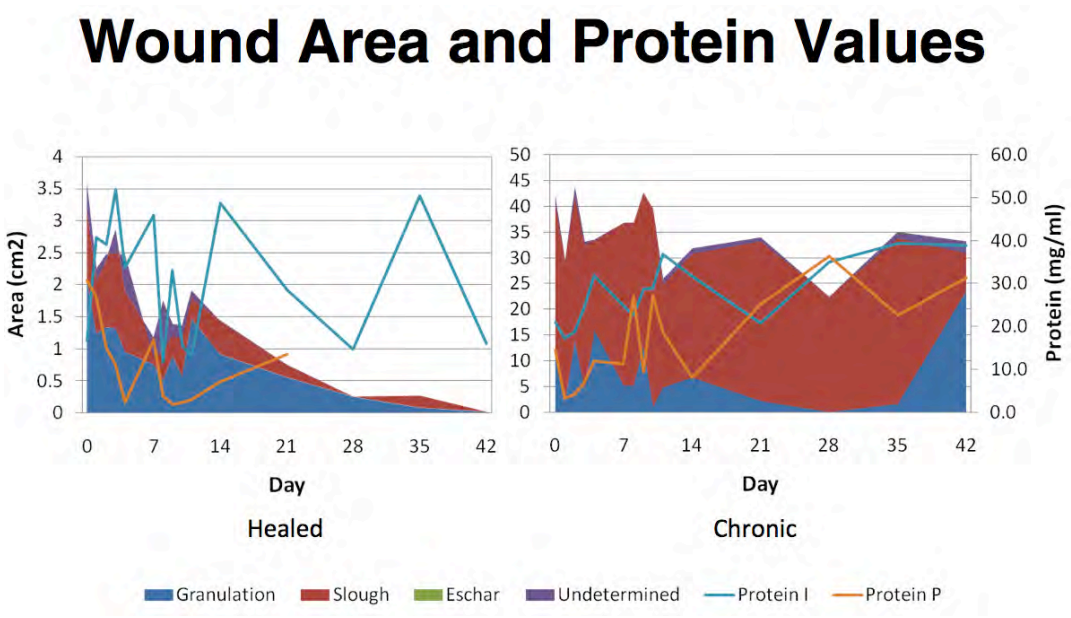


Figure 3. Tissue type and wound category versus protein present

Technical Objective 3:

To evaluate the location of the biomarkers assessed, samples were collected from both peripheral and interior locations on each wound at each time point. Antibody array data and iTRAQ data have shown differences in the molecules present and the location within the wound. Molecules associated with cell death were not found in internal sites of healed wounds, but were present in some peripheral locations. Large numbers of molecules associated with cell death present in both internal and peripheral samples from chronic wounds. S100A8 and S100A9 were upregulated in internal sites of chronic wounds only. The differences in proteins present in peripheral and internal sites of both healed and chronic wounds is being further confirmed with custom antibody arrays.

Technical Objective 4:

To identify the earliest changing biomarkers occurring in wounds which progress toward healing, changes in spot intensity are plotted versus time. Events such as a sharp decrease in the size of the wound or a change in tissue type between time points have been subjectively identified and the

iTRAQ and antibody array findings are being correlated. The time point samples for iTRAQ and array experiments are chosen in order to encompass these events.

The proteins that have been identified as unique to chronic or healed wounds are being tracked over time in the wounds and correlated with tissue type. As the biochemical profile of healing wounds is further examined it is hoped that the transition from chronic to healing will become more clearly understood Appendix A. The IPA analysis identified the Acute Phase Response Signaling Pathway as a result of the number of molecules associated with the pathway present in the samples. This inflammatory pathway has the most molecules present in the peripheral sites of chronic wounds, but several in the internal sites of these wounds and healed wounds and in the peripheral sites of healed wounds. Differences in the molecules identified over time and in location are shown in Appendix B. The inflammation stage of healing is often associated with chronic wounds and the lack of healing present in those wounds. The identification of this pathway correlates well with what is known about wound healing. The molecules identified in this present study are new to the field and of great interest as potential markers. TNF-alpha, IL-1, and IL-6 in the pathway have been previously reported in the literature, but therapeutics based on these molecules have been ineffective. The new molecules identified within the pathway may provide insight into the process of healing in chronic wounds and future treatments.

The current research is novel with respect to current published research in the field. There are no published studies characterizing of real-time surface biochemistry of pressure ulcers and no reported use of iTRAQ to analyze the proteome of pressure ulcer wound samples has been identified. Differential protein expression between healing and non-healing pressure ulcers has identified proteins, which may serve as indicators of wound healing. It is anticipated that some of the proteins identified will be significant with regard to our understanding of the healing of chronic wounds, as well as serving as potential biomarkers of healing. These biomarkers will serve as the basis of the development of an assay to predict wound outcome and may be the basis for future therapeutics developed to treat chronic wounds.

KEY RESEARCH ACCOMPLISHMENTS

- Developed methodology to map protein profiles of chronic wounds over time
- Identified proteins unique to healed wounds
- Identified proteins unique to chronic wounds
- Identified proteins unique to wound location
- Utilized IPA for analysis of iTRAQ data
- Identified pathways correlated with proteins found in wounds in each category
- Identified proteins of interest for custom arrays

REPORTABLE OUTCOMES

- "Role of Bioelectrical and Biochemical Fields in Chronic Non-Healing Wounds of People with Spinal Cord Injury", a new project based on the methodology developed with the current award, has been funded by the Ontario Neurotrauma Foundation for the period of 1/2008- 1/2010. This project will allow us to compare the biochemical profiles of pressure ulcers in people with and without spinal cord injuries. Additionally, biochemical changes after treatment with electrical stimulation will be analyzed. Enrollment is ongoing at this time.

- Edsberg LE, Fries KM, Brogan MS, Wyffels JT. Biochemical Profiles of Healing and Non-Healing Pressure Ulcers. Symposium on Advanced Wound Care and Medical Research Forum, San Diego, California, April 2008. (1st Place Oral Abstract Award)

- Abstract "Wound Surface Biochemistry of Healing and Non-Healing Pressure Ulcers", Edsberg LE, Fries KM, Brogan MS, Wyffels JT, poster presentation at the World Union of Wound Healing Societies, Third Congress, June 2008

- Invited presentation, "Potential Biomarkers of Healing and Non-Healing Pressure Ulcers", Plenary Session, 11th Annual European Pressure Ulcer Advisory Panel Meeting, Bruges, Belgium, September 2008.

- "Predicting Success in Wound Healing", Keynote Address, Care-Science and Practice, Tissue Viability Society Annual Conference, Llandudno, Wales, April 2009.

- Edsberg LE, Wyfells J. Correlation Between Protein Profiles and Tissue Types for Healing and Non-Healing Pressure Ulcers. European Wound Management Association, EWMA, Helsinki, Finland, May 2009.

CONCLUSION

The development of a methodology to identify the proteins present in chronic and healed wounds over time has been a major component in completion of the project. The utilization of iTRAQ$_{TM}$ and antibody arrays to analyze the proteins over time allows a more complete analysis of the proteins present in the wounds over the course of time.

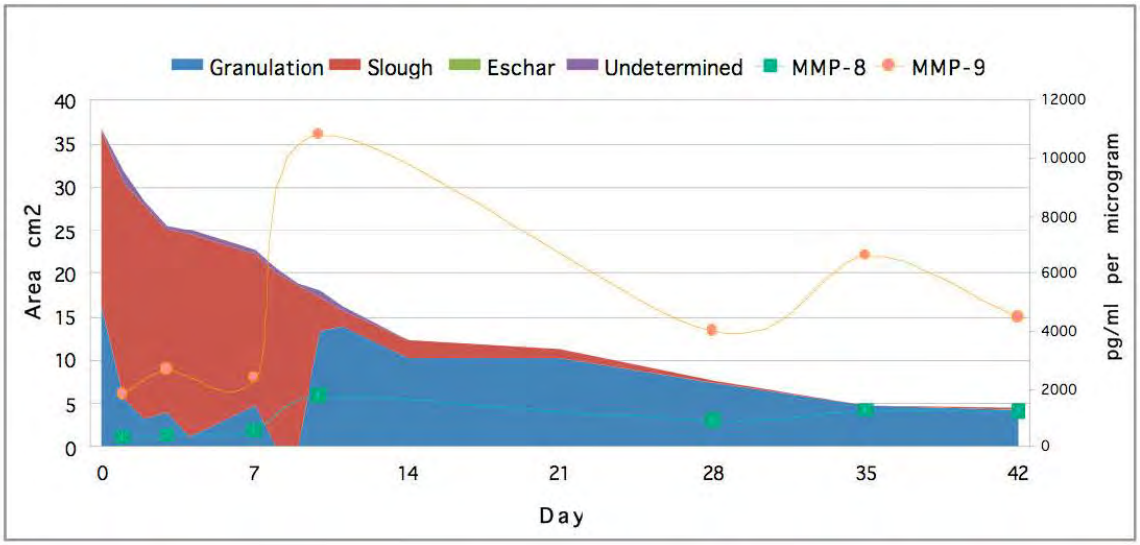
No studies have been identified using 2-D Page, iTRAQ™, and antibody arrays to characterize the environment of healed, healing, and non-healing pressure ulcers. The addition of the tissue type data will further elucidate the biochemical profile of wounds. The correlation of wound biochemistry, clinical appearance, and clinical outcome is critical to understanding of pressure ulcer healing. These findings will aid in the development of criteria for evaluating the healing process and response to treatment. Ultimately, this work may serve as a basis for profiling other types of wounds and for the development of therapies to treat wounds, which over time will decrease the suffering and deaths, as well as costs due to chronic wounds of all types.

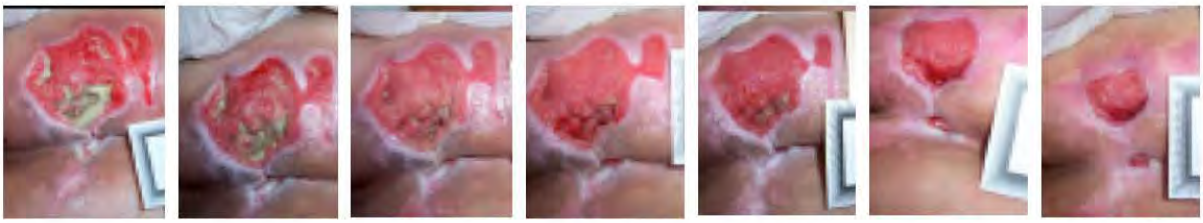
APPENDICES

Appendix A. Preliminary tissue type, wound area, and protein profile data for one wound

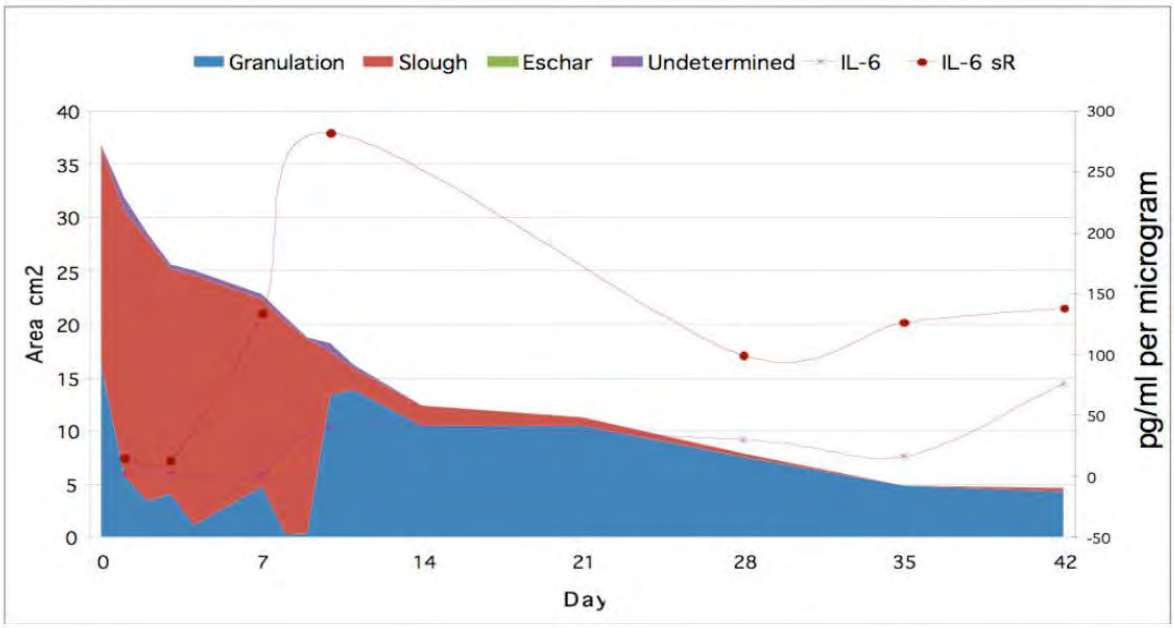
Appendix B. Acute Phase Response Signaling Pathway for a Healed and Chronic Wound

Appendix A. Preliminary tissue type, wound area, and protein profile data for one wound

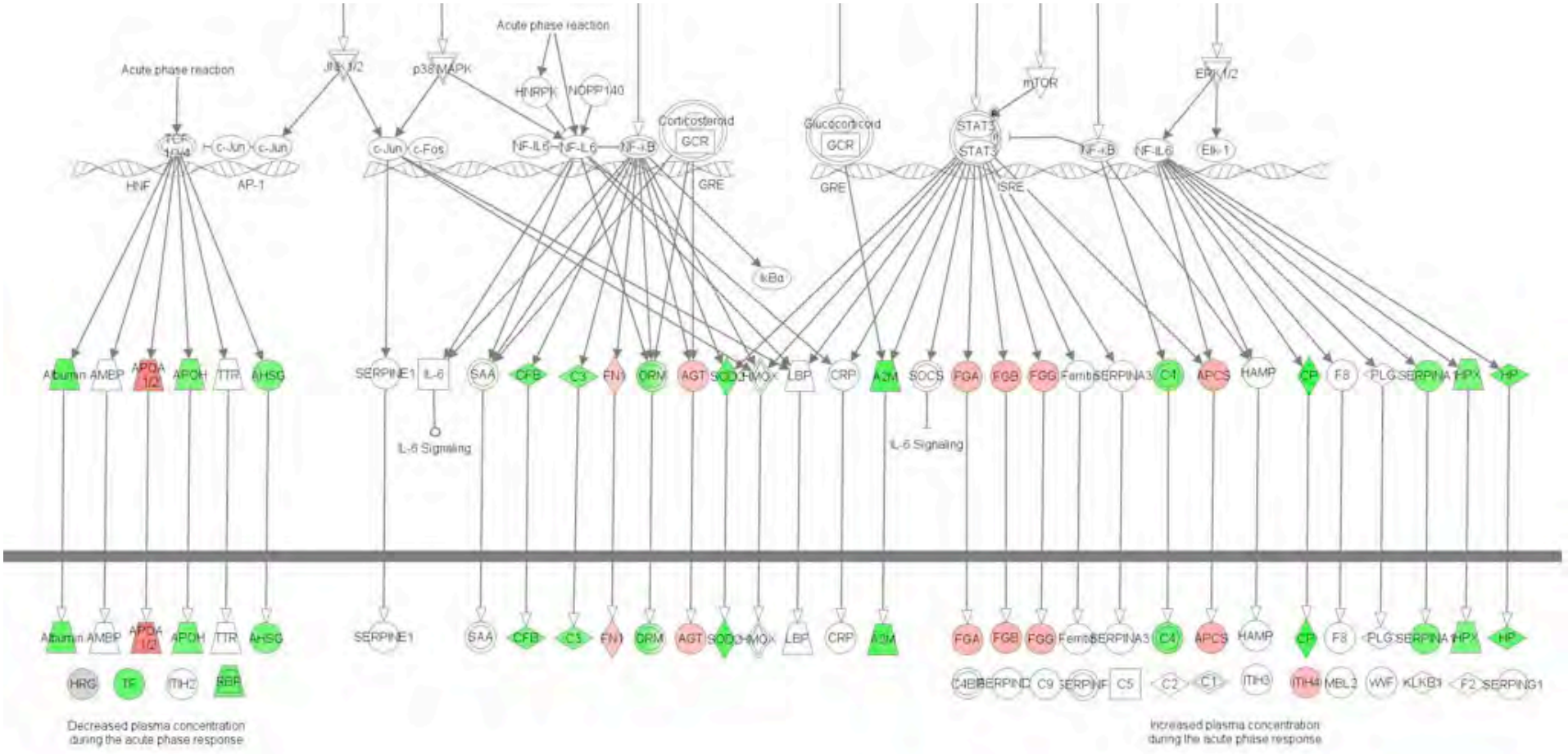




Day 0 Day 4 Day 7 Day 10 Day 14 Day 28 Day 42

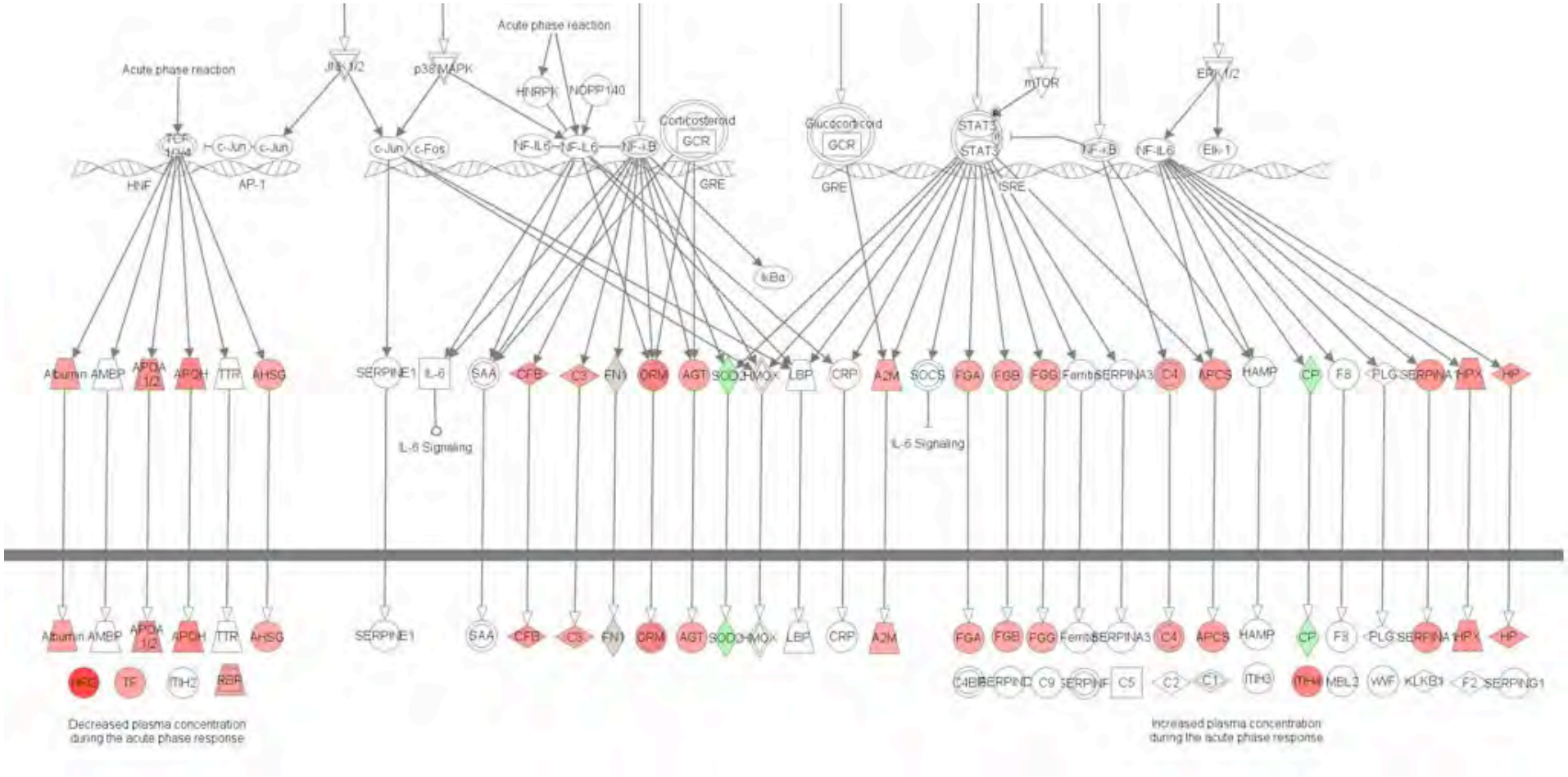


Acute Phase Response Signaling- Chronic Perimeter Day 21



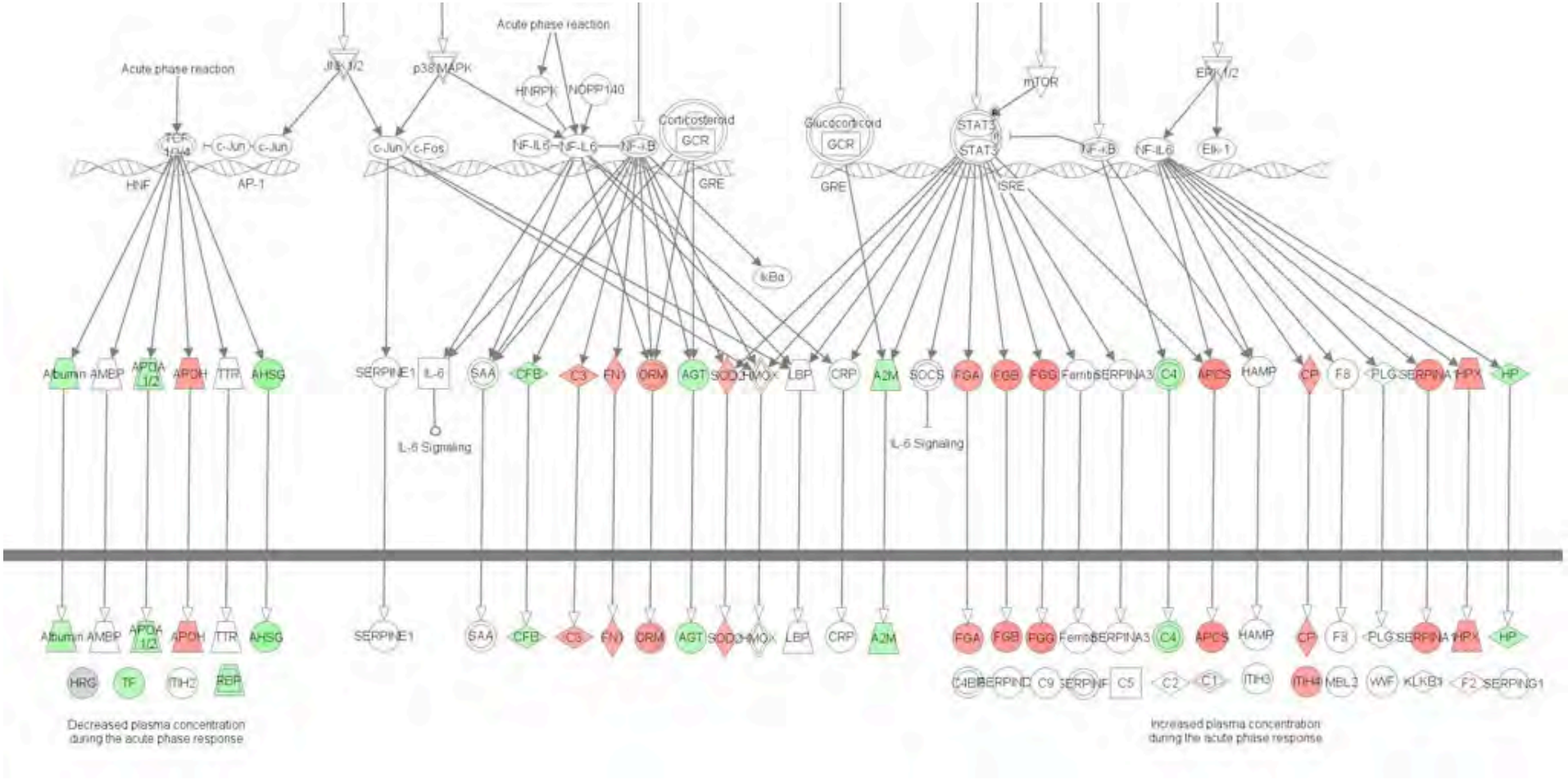
Appendix B. Acute Phase Response Signaling Pathway for a Healed and Chronic Wound

Acute Phase Response Signaling- Chronic Perimeter Day 35

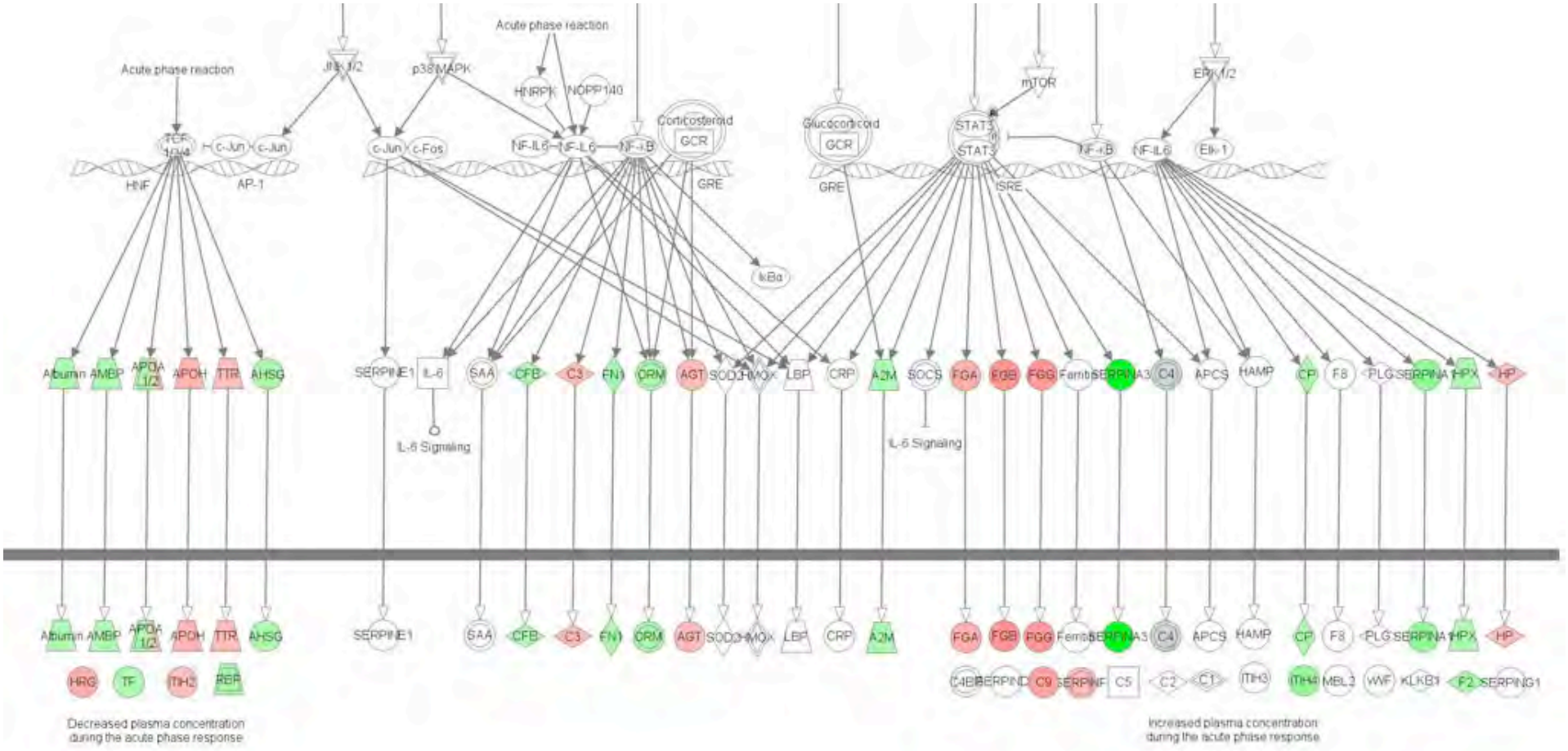


Appendix B. Acute Phase Response Signaling Pathway for a Healed and Chronic Wound

Acute Phase Response Signaling- Chronic Interior Day 35



Acute Phase Response Signaling- Healed Interior Day 14



Appendix B. Acute Phase Response Signaling Pathway for a Healed and Chronic Wound

Acute Phase Response Signaling- Healed Interior Day 21